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# Method To Detect Only Live Bacteria during PCR Amplification▽

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**Ethidium monoazide (EMA) is a DNA cross-linking agent and eukaryotic topoisomerase II poison. We previously reported that the treatment of EMA with visible light irradiation (EMA + Light) directly cleaved chromosomal DNA of *Escherichia coli* (T. Soejima, K. Iida, T. Qin, H. Taniai, M. Seki, A. Takade, and S. Yoshida, Microbiol. Immunol. 51:763–775, 2007). Herein, we report that EMA + Light randomly cleaved chromosomal DNA of heat-treated, but not live, *Listeria monocytogenes* cells within 10 min of treatment. When PCR amplified DNA that was 894 bp in size, PCR final products from $10^8$ heat-treated *L. monocytogenes* were completely suppressed by EMA + Light. When target DNA was short (113 bp), like the *hly* gene of *L. monocytogenes*, DNA amplification was not completely suppressed by EMA + Light only. Thus, we used DNA gyrase/topoisomerase IV and mammalian topoisomerase poisons (here abbreviated as T-poisons) together with EMA + Light. T-poisons could penetrate heat-treated, but not live, *L. monocytogenes* cells within 30 min to cleave chromosomal DNA by poisoning activity. The PCR product of the *hly* gene from $10^8$ heat-treated *L. monocytogenes* cells was inhibited by a combination of EMA + Light and T-poisons (EMA + Light + T-poisons), but those from live bacteria were not suppressed. As a model for clinical application to bacteremia, we tried to discriminate live and antibiotic-treated *L. monocytogenes* cells present in human blood. EMA + Light + T-poisons completely suppressed the PCR product from $10^3$ to $10^7$ antibiotic-treated *L. monocytogenes* cells but could detect $10^2$ live bacteria. Considering the prevention and control of food poisoning, this method was applied to discriminate live and heat-treated *L. monocytogenes* cells spiked into pasteurized milk. EMA + Light + T-poisons inhibited the PCR product from $10^3$ to $10^7$ heat-treated cells but could detect $10^1$ live *L. monocytogenes* cells. Our method is useful in clinical as well as food hygiene tests.**

PCR is widely used as an effective tool to detect bacteria in foods and clinical samples. The disadvantage of PCR is that it cannot discriminate dead from live bacteria. To overcome this disadvantage, reverse transcriptase PCR that targets mRNA has been used. The mRNA derived from high levels of dead bacteria ($10^4$ to $10^7$/ml), however, cannot be removed from samples, and subsequently the reverse transcriptase PCR becomes positive (31, 35). Measuring the RNA/DNA molar ratio is not sensitive enough to detect low levels of live bacteria in samples containing high levels of dead bacteria.

To discriminate live and dead bacteria by PCR, cross-linking agents such as psoralen, a methylisopsoralen derivative (4′-aminomethyl-4,5′-dimethylisopsoralen[4′-AMDMIP]), and ethidium monoazide (EMA) have been used (4, 5, 20, 23, 25, 27–29). They selectively permeate the cell walls of dead bacteria and irreversibly bind to chromosomal DNA by covalent attachment (20, 23, 27–29). It has been reported that EMA could cross-link to DNA at the rate of 1 agent per 10 to 80 bp in vitro (17). The PCR amplification of DNA from dead bacteria was inhibited by cross-linking action (23, 27–29), and the PCR signal from dead bacteria was reduced to 1/300 to 1/1,000 (23, 27, 29). It has been reported that pasteurized milk contains $10^5$ to $10^7$ cells/ml of injured/dead bacteria (1, 30). When these methods are applied to the pasteurized samples, the PCR products from injured/dead bacteria are amplified. It is very difficult to judge whether the PCR product is derived only from live bacteria in test samples.

DNase was added to cleave the chromosomal DNA of dead bacteria (21), and PCR signal intensity from dead bacteria decreased to 1/10. External DNase, however, could not completely suppress PCR products from dead bacteria, because DNase could not penetrate the cell membranes of dead bacteria due to its high molecular weight.

Developing rapid PCR methods to substitute for the culture method is a pressing matter in clinical and food hygiene tests. Most clinical samples are derived from patients administered antibiotics. Various foods have been pasteurized to kill bacteria while minimizing the denaturation of food components such as proteins. Therefore, the bacteria present in clinical samples and foods may be injured. Hyperthermophilic enzymes are reported to be maintained in the bacteria of pasteurized milk (9, 11, 36). The activities of bacterial DNA gyrase and topoisomerase IV (16) are likely to be maintained. Here, we focused on active bacterial DNA gyrase and topoisomerase IV retained in heat-treated bacteria. By utilizing the enzyme activity, we could completely suppress the PCR end products of heat-injured bacteria. There have been no reports of the inhibition of PCR products from heat-treated bacteria using DNA gyrase/topoisomerase IV poison (e.g., fluoroquinolones) and/or mammalian topoisomerase poisons (T-poisons). We

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employed EMA, which cleaves DNA after photoactivation (32), DNA gyrase/topoisomerase IV poison (8, 15, 24, 37), and mammalian topoisomerase (I and II) poisons (3, 7, 14, 18, 19, 34). We examined whether the DNA of injured/dead bacteria are cleaved by EMA with the aid of ciprofloxacin (CIN) or T-poisons and whether PCR final products from the bacteria could be completely suppressed. We used the *Listeria monocytogenes* strain, because this bacterium is important in both clinical and food hygiene applications.

## MATERIALS AND METHODS

**Reagents.** EMA (Sigma, St. Louis, MO) was used for DNA cross-linking and the DNA cleavage of bacteria. CIN, purchased from Fluka Chemie GmbH (Buchs, Germany), was dissolved in physiological saline. Ampicillin (AMP) and gentamicin (GEN) were from Sigma. Camptothecin (CAM), etoposide (ETP), ellipticine (ELP), mitoxantrone (MIT), and amsacrine (m-AMSA) were purchased from Sigma and were dissolved in dimethyl sulfoxide (DMSO).

**Bacteria and culture.** *Listeria monocytogenes* JCM 2873 was cultured at 30°C in brain heart infusion (BHI) broth (Eiken Kagaku, Tokyo, Japan). To prepare live bacterial suspensions, bacteria in the logarithmic growth phase were suspended in physiological saline. The number of live bacteria was counted by plating the bacterial suspension on Luria (L) agar after the appropriate dilution.

**Preparation of heat-treated *L. monocytogenes*.** The live bacterial suspension (1 ml) was transferred to a 1.5-ml microtube (Eppendorf, Tokyo, Japan), and the tube was soaked in a boiling water bath for 50 s. Thereafter, it was immediately chilled in an ice-water bath. This treatment simulated high-temperature, short-time pasteurization and avoided denaturing the DNA gyrase/topoisomerase IV of bacteria. The temperature of the contents was measured by a thermal sensor (TX 10; Yokogawa M C Corp., Musashino, Japan).

**Preparation of antibiotic-treated *L. monocytogenes*.** *L. monocytogenes* ($3.0 \times 10^6$ cells/ml) was treated in L broth with AMP and GEN (the final concentrations were 500 and 200 μg/ml, respectively). The suspension was incubated at 30°C for 3 weeks to bring *L. monocytogenes* completely to the injured/dead state and/or to examine whether DNA gyrase/topoisomerase IV is retained during long-term administration. The cell counts for the antibiotic-treated *L. monocytogenes* were done by a standard curve made from live bacterial counts and its optical density at 600 nm ($OD_{600}$).

**EMA treatment and visible light irradiation (EMA + Light).** EMA was dissolved in sterile water at the concentration of 1 mg/ml and filtrated through a 0.20-μm microfilter (Minisart-plus; Sartorius AG, Gottingen, Germany). After EMA was added at the concentration of 10 μg/ml to each heat- or antibiotic-treated and live bacterial suspension, it was kept at 4°C for 5 min in the dark. The suspension was then set in an ice-water bath and irradiated for 5 min with visible light (Flood PRF; 100 V, 500 W; Iwasaki Electric Co., Ltd., Tokyo, Japan) set 20 cm from the solution. The concentration of EMA was set at 10 μg/ml, because >10 μg/ml of EMA could penetrate live *L. monocytogenes* as well.

**Use of T-poisons together with EMA + Light.** After EMA + Light treatments, bacteria were washed by centrifugation. T-poisons were used to make DNA more degraded by interfering with the breakage reunion function of DNA gyrase/topoisomerase IV that is retained in the heat-treated cells. T-poisons were added to 1 ml of bacterial suspensions at volumes and concentrations (in parentheses) as follows: 8 μl of CIN (0.5 mg/ml), 10 μl of CAM (1 mg/ml), 10 μl of ETP (1 mg/ml), 5 μl of ELP (0.1 mg/ml), 10 μl of MIT (0.1 mg/ml), and 10 μl of m-AMSA (1 mg/ml). The bacterial suspensions were then incubated at 30°C for 30 min.

**CIN treatment to confirm DNA gyrase and topoisomerase IV activities retained in heat-treated *L. monocytogenes*.** Live and heat-treated *L. monocytogenes* cells were suspended in fresh BHI broth, and CIN was added at a final concentration of 20 μg/ml. They were then incubated at 30°C for 1.5, 3.5, 5, and 72 h. Simultaneously, the heat-treated bacterial suspension not treated with CIN was prepared as a control to examine the influence of DNase retained in heat-treated *L. monocytogenes* on chromosomal DNA. It was incubated for 72 h.

**Treatment of *L. monocytogenes*-added human blood.** Heparinized blood from a healthy human was cooled beforehand at 4°C. Live and antibiotic-treated *L. monocytogenes* was inoculated to the heparinized blood at concentrations of $1.8 \times 10^0$ to $1.8 \times 10^7$ cells/ml. After the sample was diluted twofold with physiological saline, 1 ml was slowly overlaid on 1.0 ml of Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in a sterilized microtube (2 ml of volume). The layers were then subjected to centrifugation at $100 \times g$ for 5 min at 4°C, and the blood plasma containing microorganism was collected. EMA + Light treatment along with the washing of the bacteria were carried out as mentioned above. When T-poison was added, the bacterial suspensions were incubated for 45 min at 30°C, followed by the same washing step. As a control, sterilized water and 0.5% (vol/vol) DMSO were used to replace EMA and T-poisons, respectively.

**Treatment of commercial milk spiked with *L. monocytogenes*.** Live and heat-treated *L. monocytogenes* ($2.2 \times 10^0$ to $2.2 \times 10^7$ cells/ml) was spiked into pasteurized milk (125°C, 2 s) in which no live *L. monocytogenes* was detected beforehand by culture and no *L. monocytogenes* was detected by PCR without EMA as described below (detection limit for live *L. monocytogenes* in milk, $2.2 \times 10^1$ cells/ml). T-poisons initially were added to 1 ml of milk inoculated with *L. monocytogenes* and then incubated at 30°C for 3 h. One milliliter of 1% Triton X-100–2 mM EDTA solution (pH 8.0) was added and centrifuged at $3,000 \times g$ for 5 min at room temperature. After the lipid and supernatant were removed, the washing step (at $15,000 \times g$ for 10 min at room temperature) was done with 2 ml of physiological saline, and then 1 ml of physiological saline was added. EMA + Light treatment and the washing of bacteria were performed as mentioned above. As a control, 0.5% DMSO and sterile water were added instead of T-poisons and EMA, respectively.

**DNA extraction from bacteria.** After 0.5 ml of 5 mM EDTA was added to bacterial pellets in a microtube (2 ml), 20 μl of achromopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan), dissolved at 5 mg/ml in 10 mM NaCl, was added and incubated at 50°C for 30 min. At this point, 0.5 ml of 10 mM Tris-HCl (pH 8.0), 20 μl of 1,250-U/ml proteinase K (Sigma, St. Louis, MO), and 400 μl of 10% (wt/vol) lauryl sulfate sodium salt solution were added one after another. The solution was incubated at 50°C overnight. The chromosomal DNA was purified by the usual phenol-chloroform extraction and ethanol precipitation. TE buffer (150 μl; 10 mM Tris-HCl, 1 mM disodium EDTA) was added to the purified DNA. The concentration of DNA was calculated from the $OD_{260}$, and purity was evaluated from the ratio of $OD_{260}/OD_{280}$. Furthermore, the usual RNase treatment was carried out successively for PCR.

**Gel electrophoresis of chromosomal DNA.** Seakem GTG agarose (FMC BioProducts, Rockland, ME) was dissolved in 0.8% Tris-acetate-EDTA (TAE) buffer. After 1 μg of purified DNA was applied to wells, electrophoresis was performed at 100 V. The λ-*Eco*T14 I digest and/or a 100-bp DNA ladder (Takara-Bio, Ohtsu, Japan) were used as DNA markers. After the gel was stained with 1 μg/ml ethidium bromide, the result was visualized with a UV transilluminator at 254 nm of UV light and recorded on Polaroid film (type 667; Nippon Polaroid, Tokyo, Japan).

**Real-time PCR.** Reactions were performed in the real-time PCR system (iCycler iQ; Bio-Rad, Hercules, CA). The fluorescence threshold was set at a value of 10× the standard deviation calculated from the fluorescence values, from 0 to 10 cycles. The first cycle for which the signals of real-time PCR amplification were above the threshold fluorescence value was set as the threshold cycle ($C_T$) value.

**Targeting genes and primers used for PCR.** The 23S rRNA gene primers to precisely discriminate live and heat-treated *L. monocytogenes* were 23S-MF (5′-ACCAGGATTTTGGCTTAGAAG-3′) and 23S-MR (5′-CACTTACCCCGACAAGGAAT-3′) (12). The length of the PCR product was 894 bp.

The listeriolysin O gene (*hly*) also was targeted to discriminate live *L. monocytogenes* from heat- and antibiotic-treated *L. monocytogenes*. The *hly* primers were *hly*-F (5′-TGCAAGTCCTAAGACGCCA-3′) and *hly*-R (5′-CACTGCATCTCCGTGGTATACTAA-3′) (22). The length of the PCR product was 113 bp.

**Amplification of 23S rRNA gene and *hly* by real-time PCR.** Fifty microliters of PCR master mix was prepared and contained 150 ng of template DNA, 5 μl of 10× Ex-*Taq* buffer (Takara-Bio), 200 μM each deoxynucleoside triphosphate (Takara-Bio), 0.25 μM of 23S rRNA gene or *hly* gene primers (Takara-Bio), 0.4× SYBR green (BMA, Rockland, ME), and 1.25 U of Ex-*Taq* polymerase (Takara-Bio).

The PCR protocol for the 23S rRNA gene of *L. monocytogenes* was 1 cycle at 4°C for 3 min, 1 cycle at 94°C for 30 s, and 40 cycles at 94°C for 20 s, 46°C for 30 s, and 72°C for 1 min. After PCR, the $T_m$ (melting-point measurement) pattern analysis of PCR product was carried out with 1 cycle at 95°C for 3 min, followed by being cooled at 60°C and heated to 95°C at the rate of 0.75°C per min.

The PCR protocol for *hly* was 1 cycle at 4°C for 3 min, 1 cycle at 94°C for 30 s, and 40 cycles at 95°C for 20 s, followed by 60°C for 1 min. After PCR, the $T_m$ pattern analysis of the PCR product was performed with the same procedures as that for the 23S rRNA gene.

In an experimental procedure applied to blood and milk, a direct PCR cocktail (G & g Science, Fukushima, Japan) was added to the bacterial pellet suspended in sterilized water (10 μl) after treatment with a combination of EMA + Light and T-poison (EMA + Light + T-poison) and successive washing, taking into consideration the simplification of DNA extraction. That is, 5 μl of bacterial

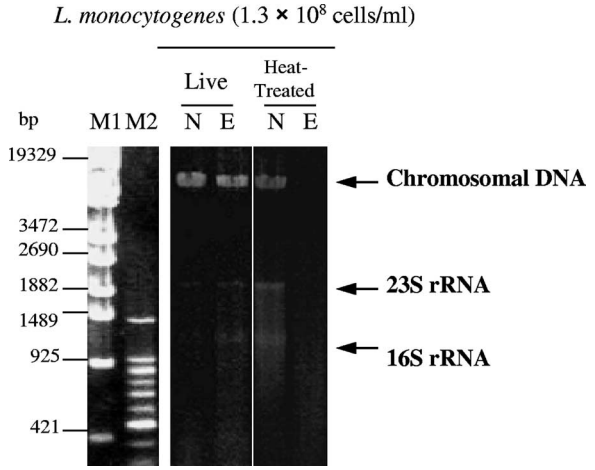


FIG. 1. Gel electrophoresis patterns of chromosomal DNA purified from live and heat-treated *L. monocytogenes* before and after EMA + Light treatment. N, no EMA + Light treatment; E, EMA + Light treatment (at 4°C in the dark for 5 min; irradiation for 5 min); M1, λ-*Eco*T14 I digest; and M2, 100-bp DNA ladder. The experiments for DNA extraction were performed in duplicate, and the electrophoresis patterns were the same.

suspension treated by EMA + Light + T-poison was added to 50.5 μl of PCR cocktail. The thermal cycle profile, which was the same as that of the PCR protocol for *hly*, was utilized.

**Electrophoresis of PCR final products amplified by real-time PCR.** A 0.8 or 3% agarose gel was made from the Seakem GTG agarose and TAE buffer for PCR final products from 23S rRNA gene and *hly*, respectively. The λ-*Eco*T14 I digest and 100-bp DNA ladder (Takara-Bio) were used as DNA markers. After 10 μl of PCR product was applied to the wells, it was separated at 100 V.

## RESULTS

**Time course of temperature and number of CFU of bacteria when bacterial suspension was inserted in boiling bath.** The relationship between the time of insertion into a boiling bath and the temperature of the contents was the following: 0 s, 25.0 ± 0.15°C; 27 s, 65.0 ± 0.20°C; 34 s, 70.0 ± 0.10°C; 47 s, 80.0 ± 0.15°C; 70 s, 90.0 ± 0.25°C; 90 s, 93.8 ± 0.60°C; and 120 s, 99.0 ± 0.45°C ($n = 3$). The relation of the immersion time and the viable cell counts (in $\log_{10}$ CFU/milliliter) of *L. monocytogenes* JCM 2873 was the following: 0 s, 8.1 ± 0.20 counts; 10 s, 7.5 ± 0.10 counts; 20 s, 6.1 ± 0.10 counts; 30 s, 4.7 ± 0.15 counts; 40 s, 2.4 ± 0.10 counts; and 50 s, no counts ($n = 3$). The detection limit was 5 CFU/ml. Insertion for 50 s offered a condition similar to that of high-temperature, short-time pasteurization; that is, 72 to 75°C for 15 to 16 s.

**Effects of EMA on the cleavage of chromosomal DNA of heat-treated and live *L. monocytogenes*.** Live and heat-treated cells of *L. monocytogenes* were treated with EMA + Light (4°C for 5 min in the dark; irradiation time, 5 min), and chromosomal DNA was purified. The gel electrophoresis patterns are shown in Fig. 1. When EMA + Light was performed on live bacteria, long fragments near 19,329 bp (derived from chromosomal DNA, because the band is fuzzy on the lower site, i.e., the anode side of the gel) were detected. As for the heat-treated *L. monocytogenes*, the long fragments did not appear, but smear bands were detectable at a range of less than 1,489 bp when EMA + Light treatment was done.

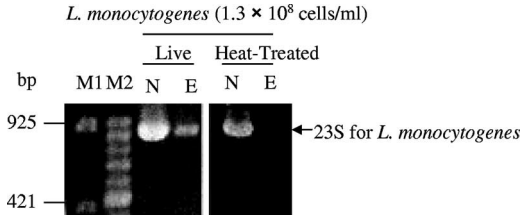


FIG. 2. Amplification results of 23S rRNA gene PCR after no treatment or EMA + Light treatment using live and heat-treated *L. monocytogenes*. The amplification results for the 23S rRNA gene (894 bp) of *L. monocytogenes* are represented. N, no EMA + Light treatment; E, EMA + Light treatment (at 4°C in the dark for 5 min; irradiation for 5 min); M1, λ-*Eco*T14 I digest; and M2, 100-bp DNA ladder. The PCR experiments were done in three replicates, and the results were the same.

**Discrimination by PCR of live and heat-treated *L. monocytogenes* after EMA + Light using the 23S rRNA gene.** PCR was performed to target the 23S rRNA gene of *L. monocytogenes* after being treated with EMA + Light. The PCR band from live *L. monocytogenes* was observed after EMA + Light treatment, but that from heat-treated cells was not observed by EMA + Light (Fig. 2). Evidently, the discrimination of live from heat-treated *L. monocytogenes* could be done.

**Discrimination of live and heat-treated *L. monocytogenes* by targeting short DNA.** When PCR targets pathogenic bacteria, short genes specific for the organism often are amplified. Our method tested whether the *hly* gene (113 bp) can be used for discrimination between live and heat-treated *L. monocytogenes*. The PCR final product from heat-treated cells was not suppressed after processing by EMA + Light; thus, discrimination between live and heat-treated *L. monocytogenes* was not successful (Fig. 3, lanes N and E). Therefore, combination methods (EMA + Light + T-poison) were performed (Fig. 3). When ETP, MIT, and m-AMSA were treated at 30°C for 30 min after EMA + Light treatment, the PCR final products from heat-treated cells were greatly inhibited, although those from live cells were not.

**Involvement of DNA gyrase/topoisomerase IV in DNA cleavage.** T-poisons impair DNA activity by accelerating the forward rate (breakage) and inhibiting the reunion of the breakage reunion activity of topoisomerases. CIN is one T-poison. The effect of CIN on the cleavage of chromosomal DNA was examined using live and heat-treated *L. monocytogenes*. The results for live and heat-treated *L. monocytogenes* are shown in Fig. 4 in CIN (+) lanes. When live *L. monocytogenes* was treated with CIN, the level of long fragments decreased time dependently during 0 to 3.5 h of incubation. The intensity of long fragments increased at 5 h but decreased to near the detection limit at 72 h. Heat-treated *L. monocytogenes* next was incubated at 30°C for 72 h with and without CIN. With CIN, the band intensity of long fragments decreased in a time-dependent manner and was near the detection limit at 72 h. Without CIN, the band intensity of long fragments (near 19,329 bp) did not decrease. These results show that DNA gyrase/topoisomerase IV is active in heat-treated *L. monocytogenes*, because T-poisons work only when topoisomerase is active. The results also imply that the influence of DNase

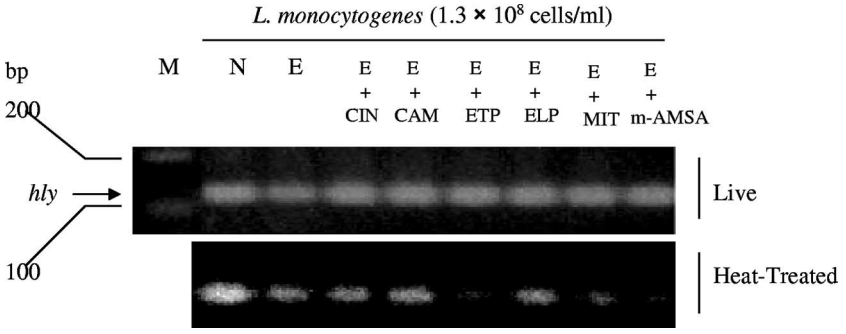


FIG. 3. Amplification results of PCR that targeted the *hly* gene after the combination treatment of EMA + Light and CIN/mammalian topoisomerase (I and II) poisons on live and heat-treated *L. monocytogenes*. The targeted *hly* gene was 113 bp. T-poison treatments were carried out for 30 min after EMA + Light treatment. N, no EMA + Light treatment; E, EMA + Light treatment (at 4°C in the dark for 5 min; irradiation for 5 min); M, 100-bp DNA ladder; CIN, 4 μg/ml CIN; CAM, 10 μg/ml CAM; ETP, 10 μg/ml ETP; ELP, 0.5 μg/ml ELP; MIT, 1 μg/ml MIT; and m-AMSA, 10 μg/ml m-AMSA. The concentrations represent final concentrations. The experiments were carried out in triplicate, and the electrophoresis images were the same.

retained in heat-treated *L. monocytogenes* on DNA cleavage is minimal.

**Discrimination of live and heat-treated *L. monocytogenes* by CIN using real-time PCR that targeted the 23S rRNA gene.** In a comparison of the thickness of bands in gels (Fig. 3), we cannot see the additive effect of CIN on EMA + Light treatment. To make the slight difference in discrimination power more distinct, the effect of CIN on the degree of PCR suppression was evaluated by the $C_T$ value of real-time PCR. The higher the $C_T$ value, the greater the PCR inhibition. $\Delta C_T$ means the degree of PCR suppression, which is represented as $C_T$ (at treatment time) minus $C_T$ (at 0 h). For live cells, the $\Delta C_T$ (at 0 to 72 h) was 0.0 ± 0.00 (at 0 h), 0.0 ± 0.00 (at 1.5 h), 1.6 ± 0.10 (at 3.5 h), 1.0 ± 0.08 (at 5 h), and 2.9 ± 0.10 (at 72 h) (means ± standard deviation; $n = 3$). As for heat-treated *L. monocytogenes*, the $\Delta C_T$ (at 0 to 72 h) was 0.0 ± 0.00 (at 0 h), 0.0 ± 0.00 (at 1.5 h), 1.0 ± 0.10 (at 3.5 h), 1.4 ± 0.10 (at 5 h), and 6.1 ± 0.15 (at 72 h).

The $\Delta C_T$ value of 2.9 ± 0.01 after 72 h of CIN treatment of live organisms was significantly different from the $\Delta C_T$ value (6.1 ± 0.15) of heat-treated cells ($P < 0.05$ by the $t$ test). Hence, the degree of PCR suppression was greater for heat-treated cells than for live *L. monocytogenes* by CIN treatment for 72 h.

**Detection limit of live *L. monocytogenes* in human blood with PCR that targeted the *hly* gene.** Figure 5A shows the results of detection for live *L. monocytogenes* inoculated into healthy human blood using PCR that targeted the *hly* gene. The PCR bands stemming from $1.8 \times 10^2$ to $1.8 \times 10^7$ cells/ml of *L. monocytogenes* in blood were clear but were not detectable from *L. monocytogenes* at concentrations of $1.8 \times 10^0$ to $1.8 \times 10^1$ cells/ml.

**PCR after EMA + Light + T-poison on live and antibiotic-treated *L. monocytogenes* inoculated into healthy human blood.** Live *L. monocytogenes* was treated with 500 μg/ml of AMP plus 200 μg/ml of GEN. Live and antibiotic-treated *L. monocytogenes* cells were inoculated into healthy human blood at concentrations of $1.8 \times 10^7$, $2.9 \times 10^4$, and $2.9 \times 10^3$ cells/ml. Figure 5B to D presents the amplified *hly* gene by PCR after EMA + Light + T-poisons (CAM, ETP, ELP, and m-AMSA) treatment. In the case of no treatment and EMA + Light ($1.8 \times 10^7$ cells/ml) (Fig. 5B), the bands of live and antibiotic-treated *L. monocytogenes* were clearly detected. However, the intensity of the bands of antibiotic-treated *L. monocytogenes* decreased to under or near the detection limit after adding CAM, ETP, or m-AMSA. The effect of ELP treatment was weak.

When $2.9 \times 10^4$ cells/ml of *L. monocytogenes* were mixed in blood (Fig. 5C), the PCR bands of live *L. monocytogenes* clearly appeared without treatment (lane N), after EMA + Light treatment (lane E), and after EMA + Light + T-poison treatment. In the case of antibiotic-treated cells, the effects of

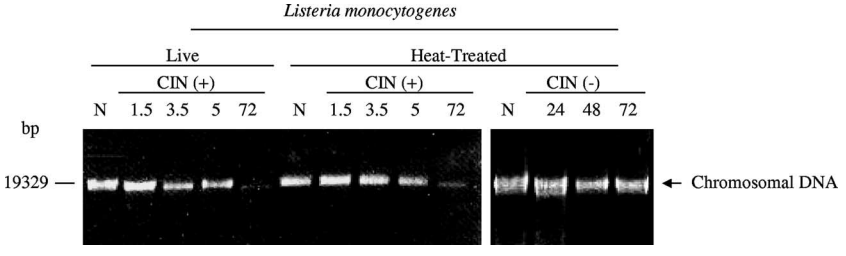


FIG. 4. Effects of CIN on the retaining activities of DNA gyrase and topoisomerase IV in heat-treated *L. monocytogenes*. The influence of CIN on the chromosomal DNA of live and heat-treated *L. monocytogenes* and the effect of DNase retained in heat-treated bacteria on the chromosomal DNA of heat-treated *L. monocytogenes* is shown. N, no treatment; CIN (+), CIN treatment. CIN (−) represents that heat-treated *L. monocytogenes* cells were incubated at 30°C for 24, 48, or 72 h without CIN. The evaluations were done in duplicate, and the same electrophoresis patterns were obtained.

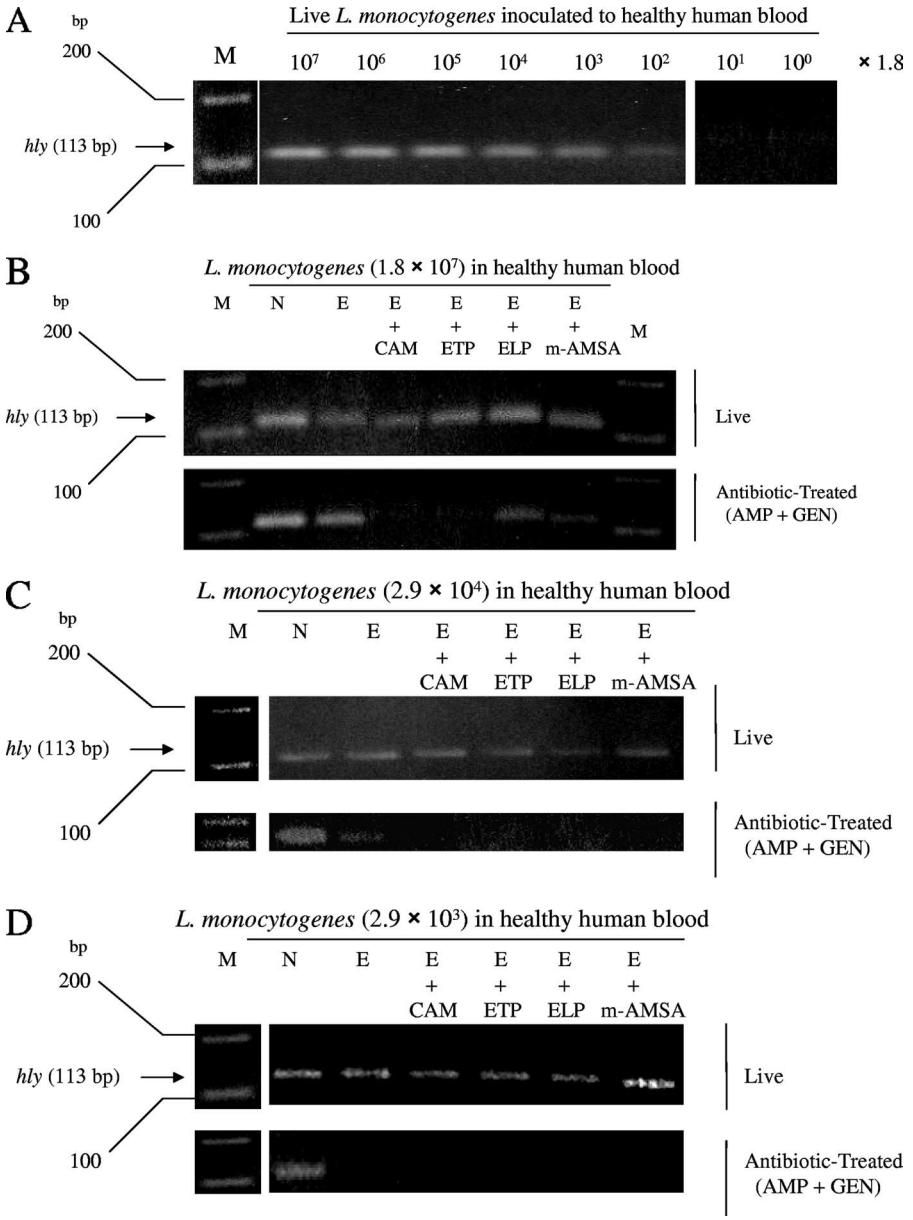


FIG. 5. Detection limit of *L. monocytogenes* and the discrimination of the live or antibiotic-treated *L. monocytogenes* cells in human blood by PCR that targeted the *hly* gene. (A) Live *L. monocytogenes* ($1.8 \times 10^0$ to $1.8 \times 10^7$ cells/ml) inoculated into heparinized healthy human blood was harvested, and the *hly* (listeriolysin O) gene (113 bp; short DNA) was targeted by PCR. (B to D) Live or antibiotic-treated *L. monocytogenes* cells were mixed in human blood, and then treatments with EMA + Light + T-poisons and PCR methods were carried out. M, 100-bp DNA ladder; *hly*, listeriolysin O gene (113 bp; short DNA) of *L. monocytogenes*; N, no treatment, as a control; E, EMA + Light (at 4°C in the dark for 5 min; irradiation for 5 min); CAM, 25 μg/ml CAM; ETP, 25 μg/ml ETP; ELP, 2.5 μg/ml ELP; and m-AMSA, 25 μg/ml m-AMSA. The concentrations represent final concentrations. Each examination was performed in duplicate, and the same results were observed.

T-poisons were clear; that is, PCR bands did not appear after EMA + Light + T-poison treatments.

Even when the *L. monocytogenes* dose was lowered to $2.9 \times 10^3$ cells/ml in blood (Fig. 5D), the effects of T-poisons were almost the same as those shown in Fig. 5C.

**Detection limit of live *L. monocytogenes*-spiked commercially available milk with PCR that targeted the *hly* gene.** Figure 6A shows the results of the detection of live *L. monocytogenes* inoculated into pasteurized commercial milk using PCR that targeted the *hly* gene. The PCR bands derived from $2.2 \times 10^1$ to $2.2 \times 10^7$ cells/ml of *L. monocytogenes* in milk were detectable. No band of *L. monocytogenes* for $2.2 \times 10^0$ cells/ml was detected, which means that the detection limit is between $2.2 \times 10^1$ and $2.2 \times 10^0$ cells/ml.

**Amplifications of PCR targeting *hly* gene after EMA + Light + T-poison to live and heat-treated *L. monocytogenes*-spiked commercial milk.** *L. monocytogenes* was inoculated into milk at concentrations of $2.2 \times 10^7$ (Fig. 6B) and $2.2 \times 10^3$ cells/ml (Fig. 6C). Figures 6B and C indicate the final products (*hly*) amplified by PCR, which was performed after EMA + Light +

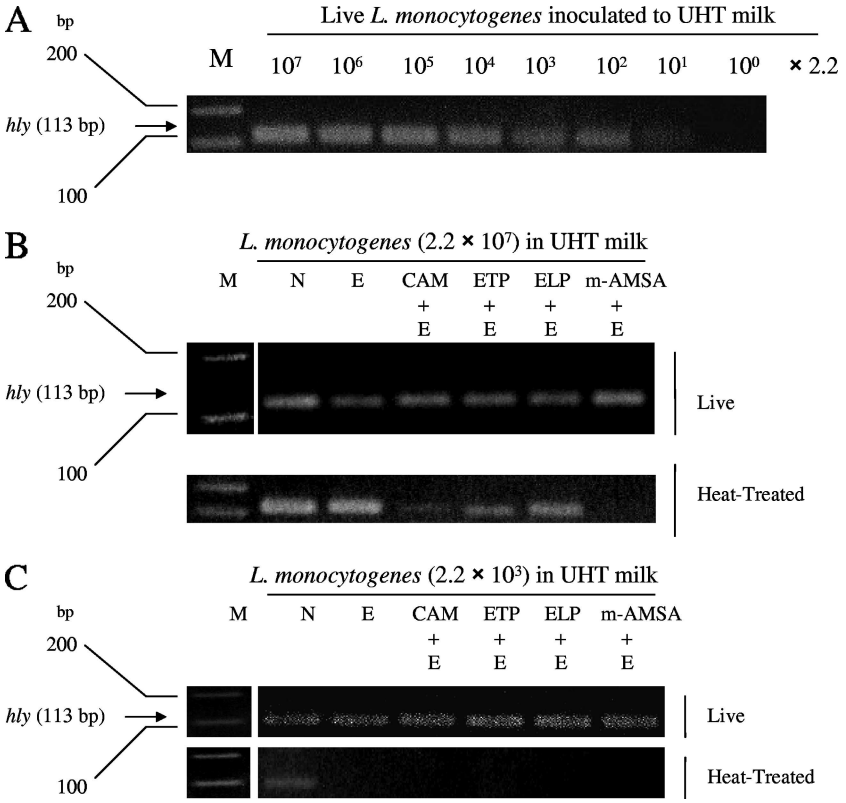


FIG. 6. Detection limit of *L. monocytogenes* and discrimination of live or heat-treated cells spiked into pasteurized milk with PCR that targeted the *hly* gene. (A) Live *L. monocytogenes* spiked into pasteurized (125°C for 2 s) commercial milk ($2.2 \times 10^0$ to $2.2 \times 10^7$ cells/ml) was recovered as bacterial pellets and supplied to a direct PCR (*hly*) cocktail. The *hly* (listeriolysin O) gene (113 bp; short DNA) was targeted by PCR. (B and C) Live or heat-treated *L. monocytogenes* was spiked in milk and then treated by EMA + Light + T-poisons and PCR methods. M, 100-bp DNA ladder; *hly*, listeriolysin O gene (113 bp; short DNA) of *L. monocytogenes*; N, no treatment, as a control; E, EMA + Light (at 4°C in the dark for 5 min; irradiation for 5 min); CAM, 25 μg/ml CAM; ETP, 25 μg/ml ETP; ELP, 2.5 μg/ml ELP; and m-AMSA, 25 μg/ml m-AMSA. The concentrations represent final concentrations. The experiments were carried out in duplicate, and the results were reproducible. UHT, ultra-high-temperature pasteurized.

T-poison (CAM, ETP, ELP, and m-AMSA) treatment on live and heat-treated *L. monocytogenes*. In the case of $2.2 \times 10^7$ cells/ml (Fig. 6B), bands of live and heat-treated *L. monocytogenes* apparently were detected both without treatment (lane N) and after EMA + Light (lane E) treatment. As for CAM + EMA + Light and m-AMSA + EMA + Light, the bands of heat-treated *L. monocytogenes* were near or under the detection limit, although the bands of live cells were clearly detected and the intensity was near that of nontreated live cells. In ETP or ELP + EMA + Light treatment, PCR bands of live and heat-treated bacteria appeared; thus, the effectiveness of the discrimination of ETP and ELP was weak when milk was used.

For $2.2 \times 10^3$ cells/ml of *L. monocytogenes* in milk (Fig. 6C), the PCR bands of live *L. monocytogenes* obviously appeared in no treatment, EMA + Light treatment, and EMA + Light + T-poison (four kinds of agents) treatment. However, PCR bands did not appear after EMA + Light and EMA + Light + T-poisons (four agents).

## DISCUSSION

Rapid PCR methods that detect only viable and culturable bacteria are required in food hygiene and clinical tests in place of the culture method. Rudi et al. (27, 29) applied EMA as a cross-linking agent to discriminate between live and dead pathogens and targeted 85-bp DNA for *Campylobacter jejuni* and the 113-bp DNA of the *hly* gene of *L. monocytogenes*. PCR signals from dead, but not live, bacteria were suppressed to 1/1,000 in dead cell counts, which means that the $C_T$ value of dead bacteria would increase by 10 cycles compared to values from no use of cross-linking EMA. Rudi et al., however, could not suppress real-time PCR final products from dead pathogens. In the case of food or clinical samples, in which the concentrations of live and dead pathogens are unknown, it is very difficult to judge whether $C_T$ values of test samples are derived from live or dead bacteria. No end product of PCR from dead cells is necessary in the factory or bedside, and the FDA requests the complete inhibition of PCR product from dead bacteria.

We have completely suppressed PCR signals from $10^7$ to $10^8$ cells of heat- and antibiotic-treated bacteria that were considered background signals in many food and clinical samples. Furthermore, PCR products derived from $10^2$ cells/ml (in blood) and $10^1$ cells/ml (in pasteurized milk) of live bacteria were detected. It is a very important consequence in clinical diagnostics and food testing that a high level of heat- and antibiotic-treated bacteria was not detected, but a low level of

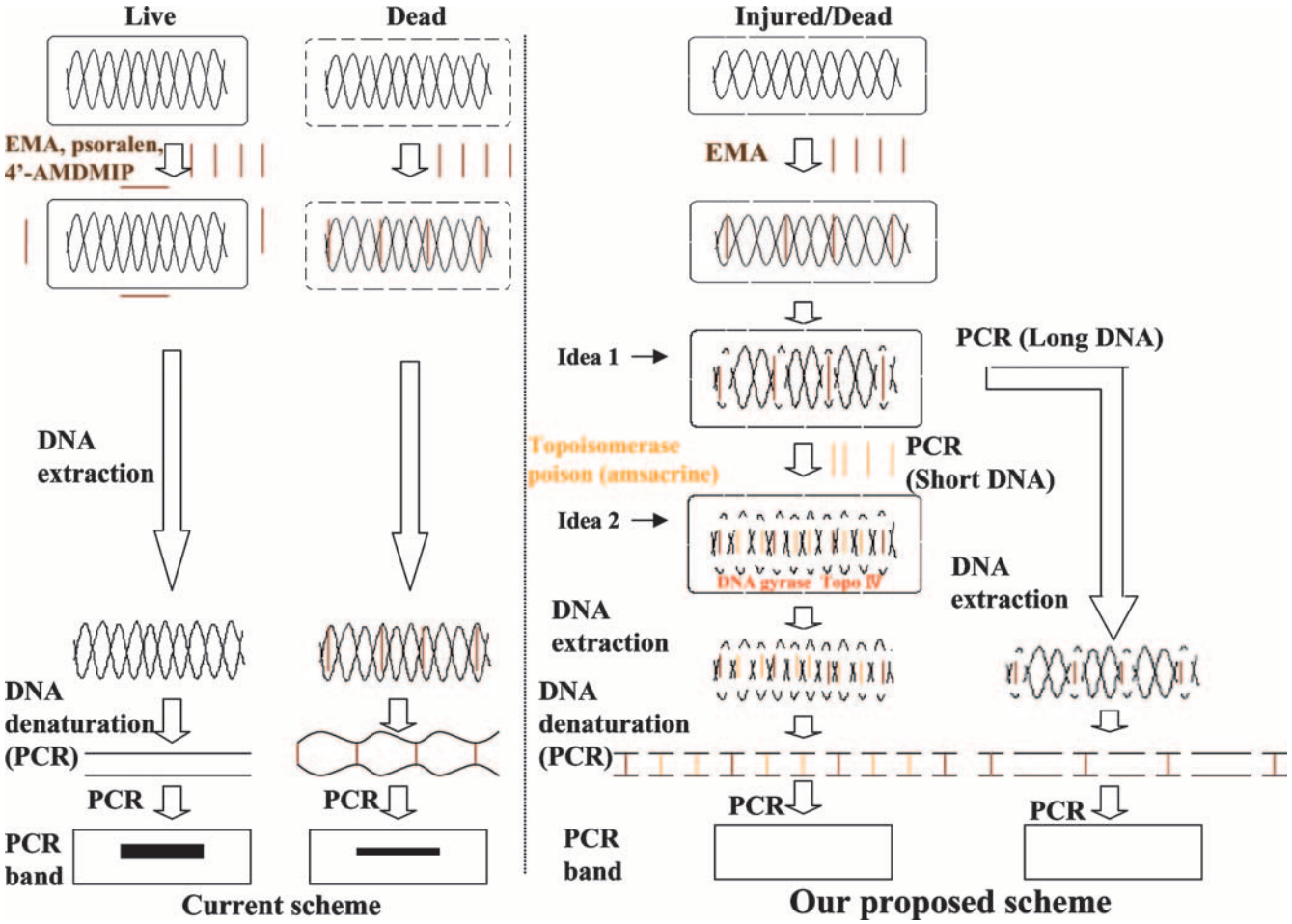


FIG. 7. (Left) Scheme for PCR suppression by EMA, psoralen, and a methylisopsoralen derivative (4′-AMDMIP) as a DNA cross-linking agent (current scheme). (Right) Scheme for PCR suppression through DNA cleavage by a new function of EMA and T-poisons containing fluoroquinolone (new scheme). Dark red bar, EMA, psoralen, or 4′-AMDMIP; yellow bar, T-poison (m-AMSA, etc.); Topo IV, bacterial topoisomerase IV. The cleavage points are represented in double-stranded DNA.

live bacteria was detectable by our PCR method. Although gel electrophoresis was used as the detection step for PCR-amplified genes in the present study, the automated $T_m$ analysis (using a real-time PCR apparatus) for PCR products described in Materials and Methods may be used, considering the rapidity, simplicity, and sensitivity to the dose of antibiotic used of live bacteria during the detection steps. If EMA + Light or EMA + Light + T-poison was combined with $T_m$ analysis after real-time PCR, $10^2$ and $10^1$ cells/ml of live bacteria in blood or milk, respectively, could be detected within 3 to 6 h.

We recently demonstrated that 1 to 10 μg/ml of EMA has the direct DNA cleavage function without mediating enzymes in bacteria, and that 50 to 100 ng/ml of EMA has the function of single-stranded breaks (32). For heat-treated *L. monocytogenes*, PCR that targeted long DNA (894 bp in the 23S rRNA gene) was suppressed by EMA + Light more than that of short DNA (113 bp in *hly*) (Fig. 2 and 3). This may be because there are more cleavage sites in long DNA. It was reported that the nanograms-per-milliliter level of EMA as a topoisomerase II poison could be cross-linked to DNA at the rate of 1 agent per 10 to 80 bp in vitro (17). In the present study, which used 10 μg/ml of EMA, direct DNA cleavage was not induced in the *hly* region of heat-treated *L. monocytogenes* (Fig. 3). We suppose that Rudi et al. (27) did not notice that PCR inhibition by EMA was not satisfactory when short DNA had been targeted. As seen above, the main cause of the failure to suppress the PCR product is that the PCR target gene was short. Even if PCR was targeted to short DNA such as that of *hly*, live *L. monocytogenes* could clearly be discriminated from heat-treated *L. monocytogenes* ($10^8$ cells/ml) by combining EMA + Light together with T-poisons (Fig. 3).

The mechanism of our method for discriminating live from dead bacteria is shown in Fig. 7. After EMA (dark red bar) penetrates heat- and antibiotic-treated cells and intercalates to the chromosomal DNA (23, 27–29), the cleavage of DNA is greatly induced by the irradiation of visible light (32). The cleavage sites are seen in double-stranded DNA. When the targeted gene is as short as *hly*, DNA cleavage sites are not likely to be contained in every bacterial cell. The cell membranes of heat- and antibiotic-treated *L. monocytogenes* are physiologically injured. Therefore, when a T-poison such as m-AMSA is added, m-AMSA randomly cross-links to chromo-

somal DNA and DNA cleavage is accelerated by inhibiting the reunion in breakage-reunion by DNA gyrase and/or topoisomerase IV retained in heat- and antibiotic-treated cells. As EMA and m-AMSA randomly cross-link, more cleavage is induced by the combination of EMA + Light + m-AMSA (Fig. 7). Hence, the disappearance of bands in Fig. 5 and 6 is thought to be due mainly to the poisoning effects of T-poisons.

The effectiveness of EMA + Light + T-poison (mainly CAM, ETP, and m-AMSA) was demonstrated in a model of bacteremia (Fig. 5). In adult bacteremia patients, the numbers of microorganisms present in blood are fewer than 10 cells/ml, and 30 ml of blood is used for culture to maximize microbial recovery. A 30-ml volume of blood could be concentrated to approximately 1 ml for PCR testing. Thus, the concentration of live bacteria would be approximately $3 \times 10^2$ cells/ml (26). In the case of bacteremia in infants, the number of bacteria existing in blood often is more than $1.0 \times 10^3$ cells/ml, but only 1 to 4.5 ml of blood should be cultured, taking into consideration the weight of the infant (13). Bacteria injured or killed by antibiotics are supposed to exist in blood together with live bacteria. In the present study, therefore, the live and antibiotic-treated *L. monocytogenes* cells were spiked into healthy human blood at the concentrations of $2.9 \times 10^4$ (Fig. 5C) and $2.9 \times 10^3$ cells/ml (Fig. 5D). On the other hand, live and antibiotic-treated *L. monocytogenes* cells were inoculated into blood at a concentration of $1.8 \times 10^7$ cells/ml, considering the presence of high levels of injured/dead bacteria in urine from urinary tract infection and sputa of tuberculosis patients given anti-tuberculosis agents (6). EMA + Light + T-poison may be effective to rapidly discriminate live from injured/dead pathogen.

Pasteurized milk contains $10^5$ to $10^7$ cells/ml of injured/dead bacteria, and approximately half of the bacteria are gram positive (1, 30). If *L. monocytogenes* was estimated to be the major contaminant, only live *L. monocytogenes* should be detected by PCR in a high level of background injured/dead *L. monocytogenes*. The effectiveness of EMA + Light and EMA + Light + T-poison (mainly m-AMSA and CAM) was tested in food hygiene tests of dairy products. Hence, as shown in Fig. 6B, live and heat-treated *L. monocytogenes* ($2.2 \times 10^7$ cells/ml) cells were inoculated into pasteurized milk. It is conceivable that the discriminating power of EMA + Light + T-poisons was inferior in milk compared to that in blood (Fig. 5B), except for the case of m-AMSA + EMA + Light. It has been reported that 2.4 to 7.5% of raw milk is contaminated by live *L. monocytogenes* (10), and live *L. monocytogenes* exists at a concentration of $2.0 \times 10^2$ cells/g in raw milk cheese (2). Hence, live and heat-treated *L. monocytogenes* ($2.2 \times 10^3$ cells/ml) also was inoculated into pasteurized milk (Fig. 6C). When *L. monocytogenes* in milk is of low concentration (live and heat-treated cells, $2.2 \times 10^3$ cells/ml), EMA + Light without T-poisons could discriminate live from heat-treated *L. monocytogenes* as well (Fig. 6C).

The verification of active DNA gyrase/topoisomerase IV retained in heat-treated bacteria is speculated as follows. As shown in Fig. 4, when live and heat-treated *L. monocytogenes* cells are treated with CIN for 72 h, the levels of long fragments derived from chromosomal DNA (close to 19,329 bp) obviously decrease. Fluoroquinolones, such as CIN, cause the inhibition of DNA synthesis and trigger cell killing by interfering with breakage reunion that is mediated by DNA gyrase (33). The reason for this phenomenon is thought to be that DNA scission is stimulated by inhibiting the reunion of breakage reunion or enhancing the forward DNA breakage rate by DNA gyrase and/or topoisomerase IV that is persistent in heat-treated and live bacteria.

Finally, our EMA + Light + T-poison method will be applied to boiled foods. When foods are treated by higher temperatures and for longer periods, the foods contain mainly dead bacteria in which no activity of DNA gyrase/topoisomerase IV is retained. In such cases, at least CAM and m-AMSA, among the T-poisons, would cross-link to chromosomal DNA and might specifically suppress PCR final products from dead bacteria (7). In this case, however, the PCR suppression is due to the cross-linking effect but not poisoning activity (7). EMA could function as a random and direct cleavage agent of chromosomal DNA with the irradiation of visible light, even if DNA gyrase and/or topoisomerase IV are completely denatured in dead cells (32).

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